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Research Article

Phylogeny and Phylogeography of *Myrmica rubra* Complex (Myrmicinae) in the Japanese Alps

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We investigated the genetic diversification of the mountain ant, *Myrmica kotokui*, in the Japanese Alps by using molecular phylogenetic analyses. *Myrmica kotokui* is widely distributed in Japan, and in the central Japanese Alps it is found only between elevations of approximately 1000 to 2000 m. We hypothesized that genetically distinct clades of this ant species might inhabit different mountain ranges in central Japan. To test this hypothesis, we reconstructed a molecular phylogeny using the DNA sequences of the mitochondrial *cytochrome oxidase I* gene and the nuclear *long-wavelength rhodopsin* gene of *M. kotokui* specimens collected from six mountain ranges in the Japanese Alps. The phylogeny showed four highly differentiated clades. However, the correspondence between the clades and morphological species was a little confusing. Two clades were composed only of *M. kotokui* specimens, whereas the other two clades were composed of multispecies, suggesting the possibility of multispecies composition of putative *M. kotokui*. The distribution pattern of these clades did not support our hypothesis of geographical differentiation, because two were distributed across all ranges, and a third was distributed in five of the six ranges. On the other hand, we found a pattern in the altitudinal distribution of the clades: one clade was distributed only at higher elevations, and the others were distributed at lower elevations. Thus, the ant clades do not show geographical segregation by mountain range, but they do show altitudinal differences.

1. Introduction

Modern molecular phylogenetic techniques have revealed that some morphological species are composed of several genetically distinct cryptic species [1]. To evaluate the biodiversity of a taxonomic group, it is essential to identify cryptic species, and biogeographical studies that determine the distributions of cryptic species are of crucial importance in conservation biology. Although many cryptic species have been found in several ant genera by using molecular phylogenetic techniques [2–5], it is difficult to identify cryptic species by using traditional morphological classification techniques alone, because intraspecific morphological variation may be greater than the interspecific variation and because convergently evolved characters may not show any morphological variation among species [3, 5]. For instance, Schlick-Steiner et al. [4] used mitochondrial DNA (mtDNA) to reconstruct the phylogeny of a *Tetramorium* ant species complex. Although the species of this complex are difficult to distinguish morphologically, by using their mtDNA phylogeny, morphology, and cuticular hydrocarbons, Schlick-Steiner et al. [4] succeeded in identifying seven cryptic species.

In the genus *Myrmica*, about 180 species are known in the Holarctic region [6]. The taxonomy of *Myrmica* is well understood in Palearctic region, and the Palearctic species are morphologically classified into some species groups [7]. In Japan, currently nine species are known, but taxonomic understanding of *Myrmica* species in Japan is not yet satisfactory [8]. *Myrmica kotokui,* which belongs to *Myrmica rubra* species group (Figure 1), is known from the Russian Far East, Korea, and Japan [9, 10] and is distributed in Japan from Hokkaido in the north to the Yakushima Islands in the south [8]. This species, which probably originated in Eurasia

Figure 1: A worker ant of *Myrmica kotokui* (a photo by T. Komatsu).

[11, 12], is adapted to a cold-temperate climate. As a result, in lower latitudes, *M. kotokui* is restricted to high elevations. For example, on Mt. Norikura in the Japanese Alps, this species is mainly found at elevations from approximately 1000 to 2000 m [13]. Thus, in the mountains of Japan, the habitat of *M. kotokui* is fragmented into several patches of mountain ranges [14], which has led us to hypothesize that *M. kotokui* might be composed of several genetically distinct clades, or cryptic species, each inhabiting a different mountain range. Moreover, this possibility is supported by the dimorphism and two social systems of *M. kotokui* that have been observed in Hokkaido. Populations of *M. kotokui* in Hokkaido have been divided into two groups according to their social structure, monogynous, or polygynous, and these two groups can also be differentiated by morphological characters of queens, namely, head width and the ratio of wing length or thorax width to head width [15–17]. Although queen body size of *M. kotokui* was significantly larger in the monogynous population than in the polygynous population, the size overlapped extensively between the two populations. Therefore, social structure of *M. kotokui* cannot be distinguished simply by queen body size [16].

The taxonomic classification of *M. kotokui* is controversial. Forel [18] described this species as a variety of *M. ruginodis,* but Collingwood [9] reclassified *M. kotokui* as a separate species. Onoyama [19] pointed out, however, that *M. kotokui* should be considered a subspecies of *M. ruginodis* because there are no remarkable morphological differences between *M. ruginodis* and *M. kotokui*. Moreover, in Japan, *M. kotokui* is likely to be confused with *M. rubra* [19]. Wetterer and Radchenko [10] also reported that in early published records from East Asia, some *M. kotokui* specimens appear to have been misidentified as *M. rubra*. *M. ruginodis* and *M. rubra* are distributed through the northern Palaearctic region, and *M. ruginodis* lives at higher latitude and altitude than *M. rubra* [20]. *M. rubra*, which is known as an alien species spreading through temperate North America [10], is infrequently discovered in Hokkaido [19].

Recently, Jansen et al. [12] reconstructed a broad-scale phylogeny of the genus *Myrmica* and confirmed that *M. kotokui* forms a genetically distinct species from *M. ruginodis* and *M. rubra* and that *M. ruginodis* is a closest relative of *M. kotokui* (also see [11]). Their phylogeny, however, was based on only two specimens of *M. kotokui* from Hokkaido; no *M. kotokui* specimens from Honshu were included. Therefore, to clarify the taxonomy of *M. kotokui* in Japan, a molecular phylogeny should be reconstructed using samples from both Honshu and Hokkaido.

For this study, we collected 72 ant colonies of *M. kotokui* in the Japanese Alps, central Honshu, and reconstructed a molecular phylogeny on the basis of the mitochondrial *cytochrome oxidase I* gene and the nuclear *long-wavelength rhodopsin* gene. Our aim was twofold: first, to clarify the phylogenetic position of *M. kotokui* among related taxa and second, to test our hypothesis that *M. kotokui* is composed of several genetic clades, each inhabiting a different mountain range.

2. Materials and Methods

2.1. Sampling and Specimens. Between June 4, and October 10, 2009, we collected 72 ant colonies of *M. kotokui* from 36 locations in six mountainous ranges of Honshu (Joshinetsu, Kita-Alps, Hijiri, Yatsugatake, Chuo-Alps, and Minami Alps) at elevations ranging from 900 to 1800 m a.s.l. Voucher specimens are deposited in the Faculty of Science, Shinshu University, Matsumoto, Japan. For the phylogenetic study, we also used as ingroups two sequences of *M. kotokui* published by Jansen et al. [12]. For outgroups, we used the *COI* and *LwRh* sequences of 11 species used by Savolainen and Vepsäläinen [21], Jansen et al. [12] and Leppänen et al. [22]: three species (*M. arisana, M. rubra,* and *M. ruginodis*) in the rubra group, which also includes *M. kotokui*; seven species (*M. formosae, M. serica, M. yamanei, M. schoedii,* Myrmica n. sp. 2, *M. indica,* and *M. weberi*) in the ritae group, a sister clade of the rubra group; one species (*M. rugosa*) of the rugosa group, a clade distant from the rubra group. In addition, for outgroups, we added four species (*M. excelsa, M. jessensis* and *M. taediosa* in lobicornis group; *M. luteola* in luteola group), which are distributed in Japan. Collection locations (latitude, longitude, and elevation) of the specimens and their GenBank accession numbers are listed in Table S1 (see Supplementary materials available online at doi:10.1155/2012/319097).

2.2. DNA Extraction, Polymerase Chain Reaction, and Sequencing. DNA was extracted from the whole body of each ant with a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocols. A mitochondrial *COI* gene and a nuclear *LwRh* gene were amplified by polymerase chain reaction (PCR) using Takara Ex Taq (Takara Bio, Shiga, Japan). The PCR primers for *COI* were mtD-6 (5′-GGA TCA CCT GAT ATA GCA TTC CC-3′) and Nancy (5′-CCC GGT AAA ATT AAA ATA TAA ACT TC-3′) [23], and that for *LwRh* were LR143F (5′-GAC AAA GTK CCA CCR GAR ATG CT-3′) [24] and LR672R (5′-CCR CAM GCW GTC ATG TTR CCT TC-3′) [12]. The PCR temperature profile used for *COI* was 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 40 s and that used for *LwRh* was 30 cycles of 98°C for 10 s, 55°C for 30 s,

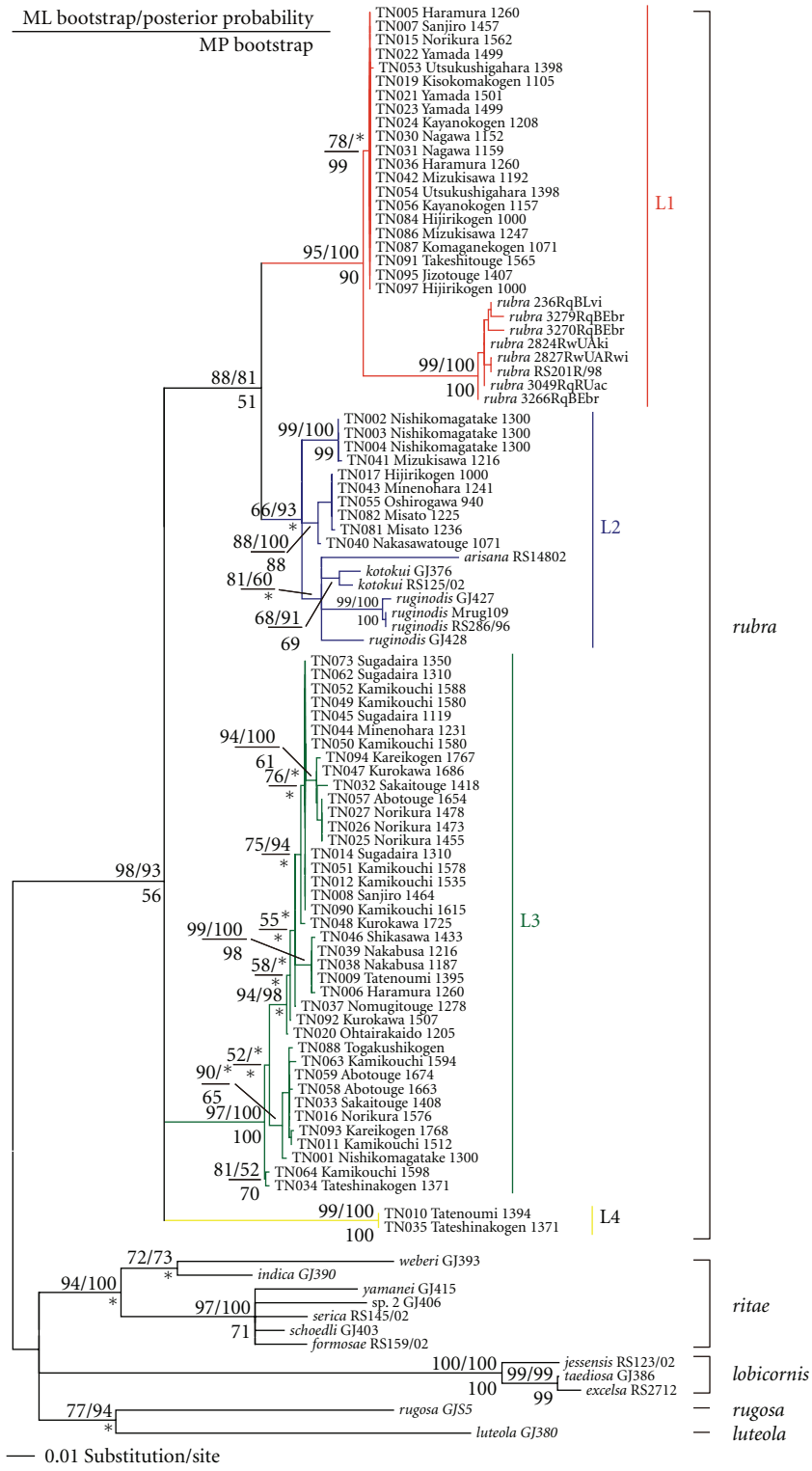

Figure 2: Maximum likelihood phylogeny of *Myrmica kotokui* estimated by using the 508 bp mitochondrial DNA sequences of *cytochrome oxidase I* and the 347 bp nuclear DNA sequences of *long-wavelength rhodopsin*. The numbers above the branches are the ML bootstrap support/Bayesian posterior probability, and those below the branches are the MP bootstrap support. An asterisk (∗) replacing a node support value indicates that the node was not recovered in the MP bootstrap or Bayesian posterior probability analysis. The sample names in the phylogeny are referred to Table S1.

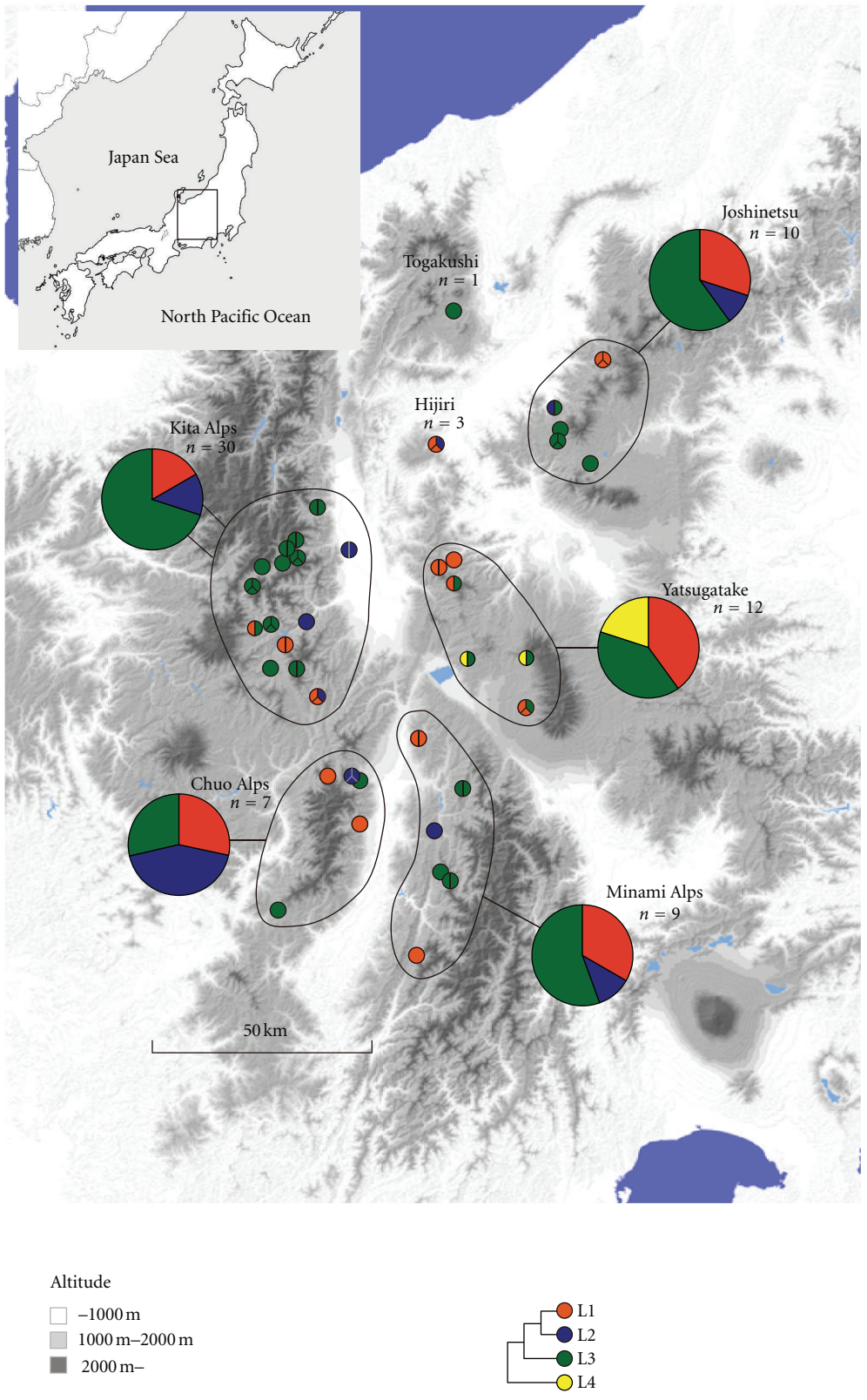

Figure 3: Geographical distribution of ant clades in the Japanese Alps. Small circles show the locations of collection sites, and divided symbols show the number of ant colonies at the same collection site. The pie charts show the proportional abundance of the clades within each mountain range with the sample size indicated.

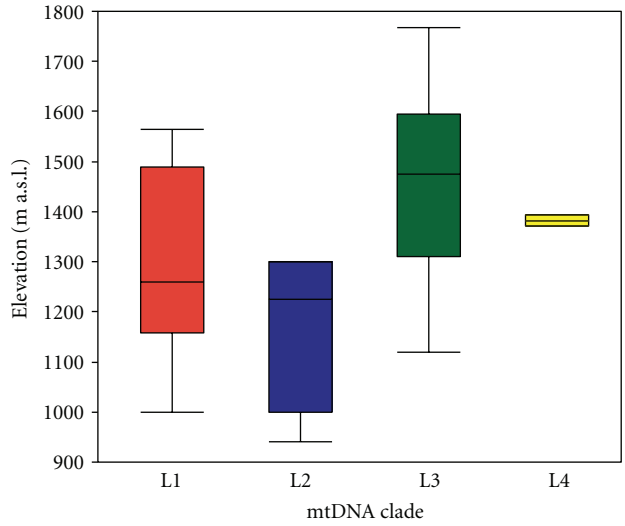

Figure 4: Altitudinal distributions of *Myrmica kotokui* clades in the Japanese Alps. The short horizontal line within each box denotes the median; the bottom and the top of each box denote the first and third quartiles, respectively, and the whiskers show the 5% and 95% limits.

and 72°C for 40 s. After amplification, the PCR products were purified with ExoSap-IT (USB, Cleveland, OH, USA). Cycle sequencing reactions for both strands were performed with a BigDye Terminator v1.1 Cycle Sequencing Kit (ABI, Weiterstadt, Germany) on an ABI 3130 Genetic Analyzer.

2.3. Sequence Alignment and Character Statistics. Mitochondrial *COI* and nuclear *LwRh* sequences were edited and aligned with SeqScape v. 2.5 (ABI, Weiterstadt, Germany). Base frequency homogeneity was tested by using the χ^2 test in the PAUP* 4.0b10 [25]. Parsimony-uninformative sites were excluded from the test. The χ^2 test did not reject the hypothesis of homogeneity of nucleotide frequencies in each pair of taxa ($P > 0.50$; Table S2). To test conflicts in phylogenetic signal among each dataset we conducted an incongruence length differences (ILDs) test (Farris et al., 1994) in PAUP* 4.0b10 with heuristic searches of 100 replicates with tree bisection and reconnection (TBR) and 10 random addition replicates. The ILD test revealed no conflict between *COI* and *LwRh* ($P = 0.79$). The degree of substitution saturation in the third codon position of the *COI* and *LwRh* sequences was assessed by plotting the transitions (*Tis*) and transversions (*Tvs*) ratios against genetic distance for each data set with the DAMBE software package by the method of Xia and Xie [26] (Figure S1). In the saturation plot analysis, the simple JC69 substitution model [27] was used instead of the J2 substitution model (*COI*) [28] or the HKY substitution model (*LwRh*) [29], which was selected in the model selections described in the next paragraph, because DAMBE does not support the J2 and the HKY substitution model. Substitution saturation at the third codon position was not detected ($P < 0.001$; Figure S1). In addition, we did not find the evidence of mitochondrial pseudogenes, called nuclear mitochondrial transfer (numts), in the mitochondrial *COI* sequences, which leads to the erroneous phylogeny [30]: there are not indels and stop codons in *COI* sequences. Following the results of the character statistics, we used all data sets and all positions in each data set for the phylogenetic analyses.

2.4. Phylogenetic Analyses. Best-fit substitution models were selected for each codon position of each gene by using Bayesian information criterion 5 (BIC5) and the Kakusan4 program [31] (Table S3). Maximum likelihood (ML) analysis was performed with TREEFINDER version October 2008 [28] and the models selected by Kakusan4. Clade support was assessed by 1000 bootstrap replications in TREEFINDER. In addition, Bayesian posterior probabilities and maximum parsimony (MP) bootstrap support were obtained with MrBayes version 3.1.2 [32] and PAUP* 4.0b10 software [25], respectively. The models selected by BIC5 were also used in the Bayesian analysis (Table S3) with the default run settings, in which two independent analyses are performed, each with four chains (one cold and three heated). The Bayesian analysis was run for 1 million generations, with sampling every 1000 generations. We assessed the log likelihood of each sampling point against generation time to identify when the Markov chains reached a stationary distribution and accordingly discarded the initial 2000 trees as burn-in. The parsimony bootstrap support was assessed with 1000 bootstrap replicates by using heuristic searches with tree bisection and reconnection (TBR) and 100 random addition replicates for each.

2.5. Statistical Analysis. Differences in the elevation range among the distributions of the ant clades were analyzed by one-way analysis of variance (ANOVA) and Tukey's multiple comparison test in the R software package [33].

3. Results and Discussion

3.1. Taxonomy of M. kotokui Based on DNA Phylogeny. Molecular phylogenies of *M. kotokui* were inferred from a total of 855 bp from the two genes (508 bp sequences of the mitochondrial *COI* gene and 347 bp sequences of the nuclear *LwRh* gene). Although there is no conflict of the phylogenetic signal between the mitochondrial *COI* gene and the nuclear *LwRh* gene, the further analyses of additional nuclear gene sequences will be required to reveal the conflict between mtDNA and nuclear DNA phylogenies due to introgression or incomplete lineage sorting [34, 35]. It is because the substitution rate of *LwRh* is much lower than that of *COI* and genetic divergences between L1 and L2 and between L3 and L4 were not detected in the analysis of the nuclear *LwRh* marker alone (data not shown).

Four independent clades were identified by the ML, MP, and Bayesian analyses (L1 to L4; Figure 2), suggesting that the single morphological species known as *M. kotokui* is composed of several putative cryptic species. However, the correspondence between the clades and morphological species was a little confusing. Two clades (L3 and L4) were composed only of *M. kotokui* specimens, whereas the other

two clades composed of multispecies: L1 was composed of *M. rubra* and *M. kotokui* specimens, and L2 was composed of *M. arisana* and *M. ruginodis* as well as *M. kotokui* specimens (Figure 2).

The possibility of multispecies composition of *M. kotokui* may account for the confused taxonomy of *M. kotokui* in Japan as reported by several studies [10, 15, 19, 36]. The absence of morphological differences between *M. kotokui* and *M. ruginodis* reported by Onoyama [19], for example, might be because *M. kotokui* specimens of the L2 clade, which is closely related to *M. ruginodis* (Figure 2), were used for the morphological comparison of the two species. Moreover, the misidentification of *M. kotokui* specimens as *M. rubra* [10, 19] may have occurred because the L1 clade of *M. kotokui* is closely related to *M. rubra* (Figure 2).

Taken at face value, it is possible that the L1 and L2 of putative *M. kotokui* are misidentification of *M. rubra* and *M. ruginodis,* respectively, and that the L3 or L4 clades, or both, should be regarded as the true species *M. kotokui*. Here, the taxonomy of *M. kotokui* cannot be clarified because detailed morphological comparisons have not been made with type material. All specimens collected from the Japanese Alps for this study were identified as *M. kotokui* by using the taxonomic key for Japanese ant species of Morisita et al. [37], which was made based on the original species description of Collingwood [9], but further morphological study of the specimens is desirable. Further multidisciplinary studies of the morphology, social structure, and cuticular hydrocarbons of *M. kotokui* and its relatives [4, 5] are also necessary to solve these taxonomic problems.

3.2. Geographical Distribution and Altitudinal Distribution. We investigated the geographical distribution of each clade to determine whether they were allopatrically isolated. We found that L1 and L3 were distributed across all six mountain ranges, L2 was distributed in all mountain ranges except Yatsugatake, and L4 (present in only two samples) was found only in Yatsugatake (Figure 3). Thus, the distribution pattern of the ant clades did not support our hypothesis of geographical genetic differentiation by mountain range. Therefore, we investigated the altitudinal distributions of the ant clades in the Japanese Alps between 900 and 1800 m a.s.l. and found that the L3 clade was distributed at a significantly higher elevation range than L1 and L2 (Figure 4; one-way ANOVA, $P < 0.001$; Tukey's multiple comparisons: L1 $\times$ L3, $P < 0.005$; L2 $\times$ L3, $P < 0.0001$). A significant difference was not detected between the L4 distribution and the other distributions because of the small L4 sample size.

These results raise the following question. Did the genetic differentiation of the ant clades occur in the Japanese Alps or did clades that had already diverged on the Eurasian continent migrate to the Japanese Alps? The differentiation of the clades may not have occurred in the Japanese Alps for two reasons: (1) no physical barrier separates altitudinal ranges in the Japanese Alps and (2) the genetic distances between the clades are too large (6.5%) to have been accumulated after the last glacial maximum. Thus, the altitudinally stratified distribution of the ant clades may reflect multiple migrations from the continent with clades adapted to different levels of coldness becoming segregated in different habitats because of the drastic environmental differences along the elevation gradient [38]. Taken together, our results suggest that a cryptic highland clade of *Myrmica* exists. We also propose that further biodiversity may be hidden in ants that live at high elevations.

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